

## DESCRIPTION

Primer and probe for detecting *Vibrio cholerae* or *Vibrio mimicus* and detection method using the same

## TECHNICAL FIELD

The present invention relates to a method for detecting, identifying, and counting *Vibrio cholerae* and *Vibrio mimicus* in food inspection, epidemiological environmental inspection, and clinical examination.

## BACKGROUND ART

*Vibrio cholerae* produces a cholera toxin after oral infection and induces violent diarrhea and vomiting. In some cases, the infectious disease bacterium may cause affected patients to die from extreme dehydration. It has been thought that *Vibrio cholerae*, being toxic to humans, is only O1 cholerae that agglutinates by the use of an anti-O1 antibody. It has also been thought that *Vibrio* other than the bacterium is classified as NAG (O1-non-agglutinable)-*Vibrio* and is not toxic to humans. However, in the Bengal district in India in 1995, a new type of *Vibrio cholerae* having no O1 antigen that causes symptoms similar to those caused by conventional cholerae was isolated, revealing that the new *Vibrio cholerae* strain has a new O-antigen referred to as O139. This strain is referred to as the Bengal strain and has been revealed to have a cholerae toxin-producing gene similar to that of conventional O1 cholerae, which produces a toxin and induces cholera. Accordingly, it has been shown that *Vibrio* other than O1 cholerae can infect humans and cause cholera. Actually, *Vibrio cholerae* strains having cholera toxin genes are present, in addition to O1 and O139 strains. Such strains induce cholera in humans. However, symptoms due to *Vibrio cholerae* strains other than the O1 and O139 strains are not treated as infectious diseases administratively.

In the meantime, a non-saccharolytic strain has been rarely detected among non-O1 strains of *Vibrio cholerae*. Davis et al., have examined DNA homology of the strain with *Vibrio cholerae*, thereby showing that the strain is somewhat different from *Vibrio cholerae* and designating the strain as *Vibrio mimicus*. It has been reported that *Vibrio mimicus* is a strain very closely related to *Vibrio cholerae*. Specifically, except saccharolytic ability (*Vibrio cholerae* is positive and *Vibrio mimicus* is negative), the biochemical properties of the two species are very similar (J. Clin. Microbiol. 14, 631-639, 1981) (non-patent document 1). It is known that among *Vibrio mimicus* there are strains having a cholera toxin gene similar to that of *Vibrio cholerae* (Microbiol Immunol 1998, 42, 823-828) (non-patent document 2). *Vibrio mimicus* may thus induce symptoms similar to those of cholera.

Furthermore, among *Vibrio cholerae* having no cholera toxin genes, strains that have been reported to be present are: strains having a thermostable direct hemolysin (tdh) gene (Appl Environ Microbiol 52, 1218-20, 1986) (non-patent document 3) that is a pathogenic factor of *Vibrio parahaemolyticus*; and a strain producing a thermostable enterotoxin of *Escherichia coli* (J. Clin. Invest. 85: 697-705, 1990) (non-patent document 4). They can cause diarrhea and the like. Similarly, among *Vibrio mimicus*, a strain having a tdh gene has been reported to be present (FEMS Microbiol 59: 319-23, 1990) (non-patent document 5), which can cause diarrhea and the like even when it does not produce any cholera toxin.

As described above, a case in which *Vibrio cholerae* having serotype of O1 or O139 (Bengal) and producing a cholera toxin is detected is a subject to measures specified in the "Law Concerning Prevention of Infection of Infectious Diseases and Patients with Infectious Diseases." Moreover, there are also cholera toxin-producing strains among *Vibrio cholerae* other than O1 cholerae and Bengal cholerae, which induce symptoms similar to those of cholera. Furthermore, strains producing no cholera toxins do not cause cholera. However,

this does not mean that they are non-pathogenic. Such strains induce acute gastroenteritis, diarrhea, and the like by producing toxins other than the cholera toxin. Therefore, it is required to rapidly and precisely detect the *Vibrio cholerae* bacterial group.

Furthermore, in the case of *Vibrio mimicus*, there are strains producing a cholera toxin that can cause cholera and strains causing acute gastroenteritis, diarrhea, and the like through the production of toxins other than the cholera toxin. Therefore, it is also required to rapidly and precisely detect the *Vibrio mimicus* bacterial group.

Conventional methods of examination using biochemical techniques require skilled technology and much effort and time. To compensate for such deficiencies, precise, rapid, and convenient methods of examination using genes have been attempted for detecting or identifying *Vibrio cholerae* and *Vibrio mimicus*.

Primers for detecting the gene encoding the cholera toxin that causes cholera are already known (J. Biol. Chem. 1983, 258, 13722-13726) (non-patent document 6). However, it is impossible to detect *Vibrio cholerae* and *Vibrio mimicus*, which do not have any cholera toxin gene but which produce other toxins through the use of such primers.

In the meantime, when *Vibrio cholerae* and *Vibrio mimicus* are compared in terms of the full-length nucleotide sequence of a 16S rRNA gene, only 6 among 1,456 nucleotides are found to differ (Int. J. Syst. Bacteriol. 44, 416-426, 1994) (non-patent document 7). Thus, it is impossible to clearly distinguish between the two. Accordingly, there is a report wherein *Vibrio cholerae* and *Vibrio mimicus* were compared in terms of the 16S-23S rRNA intergenic spacer region so as to show that they can be classified and primers with which only *Vibrio cholerae* can be detected were produced (Appl. Environ. Microbiol. 65, 2202-2208, 1999) (non-patent document 8). However, there is also a report wherein *Vibrio mimicus* was detected by mistake when the primers were used

(Appl. Environ. Microbiol. 67, 2360-2364 2001) (non-patent document 9).

## SUMMARY OF THE INVENTION

As described above, it has been impossible to detect with high accuracy *Vibrio cholerae* and *Vibrio mimicus* by the existing genetic detection methods.

What is common among conventional genetic screening methods is that they ignore the fact that a bacterial "species" is a population containing genetic diversity. The use of a nucleotide sequence of a single bacterial strain that is inferred to be a member of a bacterial population as a common sequence for such population or a sequence representing such population is very dangerous in terms of molecular evolutionary characteristics of genes, which rapidly accumulate neutral mutations. Specifically, there is concern that such use could cause misidentification such that a bacterial strain to be detected could not be detected because of inhibited amplification due to a slight mutation in a primer region. There is also concern that a closely related strain not to be detected would be detected because of insufficient primer specificity. Hence, it has been required to produce a high-performance and specific gene amplification primer for detecting, identifying, or quantifying *Vibrio cholerae* and *Vibrio mimicus* and a gene amplification primer for specifically detecting, identifying, or quantifying *Vibrio cholerae* and *Vibrio mimicus*, which have proven backgrounds of specificity, low possibility of misidentification, and practically sufficient amplification efficiency and amplification specificity.

To produce a method for specifically detecting a gene of a bacterial phylogenetic group, there is a need to collect as many nucleotide sequences as possible from a biological group to be detected and from biological groups phylogenetically close to these groups. Moreover, a gene targeted for specific detection should have a sufficiently unique nucleotide sequence so that it is distinguishable from the most closely related organisms. In order to satisfy these conditions, a target gene must have a sufficiently rapid rate of evolution.

In addition, as in the case of a gene that is transmitted horizontally at a high frequency (e.g., a toxin gene of *Vibrio parahaemolyticus*), a gene that exists independently of the phylogenetic line cannot be used. Proteins encoded by a *gyrB* gene and an *rpoD* gene used as targets in the present invention are essential for survival. Because of this reason, these genes are hardly transmitted horizontally and have appropriate rates of evolution, so that they are preferably used for bacterial phylogenetic analysis (Int. J. Syst. Bacteriol. 1998, 48, 813-819; Int. J. Syst. Bacteriol. 1999, 49, 87-95). We have already developed a convenient method for determining a nucleotide sequence that involves subjecting the *gyrB* gene and the *rpoD* gene to the PCR direct sequencing method (JP Patent Publication (Kokai) Nos. 07-213229 A (1995) and 08-256798 A (1996)). Furthermore, we have already isolated large quantities of *Vibrio cholerae*. In the meantime, regarding a known stock strain of *Vibrio mimicus* and those reported to be closely related to this bacterium according to analysis based on a 16S rRNA sequence, *Listonella anguillarum*, *V. ordalii*, *V. diazotrophicus*, *V. vulnificus*, *V. navarrensis*, *V. metschnikovii*, and *V. cincinnatiensis* (International Journal of Systematic and Evolutionary Microbiology 51, 1449-1456 (2001)), we have analyzed the partial nucleotide sequences of the *gyrB* and *rpoD* genes of bacterial strains listed in Table 1 and conducted molecular phylogenetic analysis based on such sequences, thereby revealing the phylogenetic relationship.

Table 1. Strains used

<i>V. cholerae</i>	29 strains	Clinically isolated strains	29 strains
<i>V. mimicus</i>	4 strains	ATCC 33653 T ATCC 33654 ATCC 33655 ATCC 700326	
Other stock strains of the genus <i>Vibrio</i>	52 strains		
<i>V. vulnificus</i>	32 strains	ATCC 27562 T ATCC 29306 ATCC 29307 ATCC 33147 ATCC 33148 ATCC 33149 ATCC 33814 ATCC 33815 ATCC 33816 ATCC 33817 ATCC 43382 ATCC BAA-86 ATCC BAA-87 ATCC BAA-88 ATCC BAA-89 ATCC BAA-90 JCM 3726 JCM 3727 JCM 3728 JCM 3729 JCM 3730 JCM 3731	
		Strains isolated from environment	10 strains
<i>V. diazotrophicus</i>		ATCC 33466 T	
<i>V. navarrensis</i>		ATCC 51183 T	
<i>V. metschnikovii</i>		ATCC 700040 T	
<i>V. cincinnatiensis</i>		ATCC 35912 T	
<i>V. ordalii</i>		NCIMB 2167 T	
<i>Listonella anguillarum</i>		NCIMB 6 T	
<i>V. hollisae</i>		ATCC 33564 T	
<i>V. alginolyticus</i>		IFO 15630 T	
<i>V. campbellii</i>		IFO 15631 T	
<i>V. carchariae</i>		IFO 15632 T	
<i>V. harveyi</i>		IFO 15634 T	
<i>V. nereis</i>		IFO 15637 T	

<i>V. parahaemolyticus</i>		IFO 12711 T	
<i>V. proteolyticus</i>		IFO 13287 T	
<i>V. tubiashii</i>		IFO 15644 T	
<i>Vibrio. sp</i>	5 strains	Strains isolated from food	
			Total 85 strains

Specifically, after the test strains had been subjected to enrichment culture using brain heart infusion media supplemented with 2% NaCl, chromosomal DNA was extracted using a PUREGENE DNA Isolation Kit (Gentra SYSTEMS). Using the extracted DNA as a template, a *gyrB* gene fragment with a length of approximately 900 bp (a region corresponding to positions 331 to 1212 of the nucleotide sequence or positions 111 to 404 of the amino acid sequence of *Escherichia coli* strain K-12) was amplified by PCR using *gyrB* universal amplification primers UP-1E and AprU. Furthermore, similarly, an *rpoD* gene fragment with a length of approximately 800 bp (a region corresponding to positions 334 to 1125 of the nucleotide sequence and positions 112 to 375 of the amino acid sequence of *Escherichia coli* strain K-12) was amplified by PCR using *rpoD* universal amplification primers 70F-M13 and 70R-M13. The obtained PCR product was subjected to 1% agarose gel electrophoresis, and then stained with ethidium bromide. Amplified products were confirmed to be present under UV irradiation, and then purified using a Wizard PCR Preps DNA Purification System (Promega), thereby preparing templates for sequence reaction. Cycle sequence reaction was conducted using as primers M13R and M13-21 sequences previously added to the universal primers and an ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems). The nucleotide sequences were analyzed using an ABI PRISM 310 Genetic Analyzer (PE Applied Biosystems). Upon molecular phylogenetic analysis using the determined nucleotide sequences, to more precisely understand closely related species of *Vibrio cholera* and *mimicus*, analysis was conducted after linking partial

sequences of the *gyrB* and *rpoD* genes. The obtained nucleotide sequences were subjected to multiple alignment analysis using a Clustal W computer program. Subsequently, through the use of a PHYLIP computer program package, a molecular phylogenetic tree was produced by the neighbor-joining method based on genetic distance calculated using Kimura's 2-parameter model. As a result, it was shown that *Vibrio cholerae* and *mimicus* belong to mono phyletic line different from those of other bacteria of the genus *Vibrio*. Specifically, it was suggested that *Vibrio cholerae* and *mimicus* each forms an independent mono phyletic group (Fig. 1). Hence, to establish a genetic screening method that can detect only *Vibrio cholerae* and *mimicus* bacterial groups, firstly differences in nucleotide sequences among closely related species were revealed. That is, nucleotide positions that are conserved within the *Vibrio cholerae* and *mimicus* groups but differ from other bacteria of the genus *Vibrio* were identified. Specifically, consensus sequences of the phylogenetic groups to which *Vibrio cholerae* and *mimicus* belong were found and compared with consensus sequences of C1 to C3 clusters in Fig. 1 determined to be closely related to this phylogenetic line based on the results of molecular phylogenetic analysis. Thus, a phylogenetically specific information map was produced (Figs. 2 and 3). The following positions are specific to the phylogenetic line to which *Vibrio cholerae* and *mimicus* belong: as shown in Fig. 2, in the case of the *gyrB* gene, positions (hereinafter also referred to as nucleotide number) 21, 96, 107, 126, 153, 190, 258, 270, 279, 285, 357, 543, 552, 557, 600, 690, 702, 714, 729, 733, 734, 759, 771, 782, 786, 792, 795, and 885 of SEQ ID NO: 1 in the sequence listing; and as shown in Fig. 3, in the case of the *rpoD* gene, positions 3, 27, 66, 67, 75, 90, 117, 123, 141, 144, 177, 178, 180, 186, 223, 227, 228, 231, 250, 251, 255, 257, 259, 264, 300, 301, 302, 303, 305, 313, 314, 350, 351, 362, 369, 373, 374, 380, 390, 400, 402, 409, 410, 415, 416, 423, 427, 433, 444, 447, 504, 510, 513, 543, 556, 558, 618, 638, 649, 663, 685, 711, 747, 757, 762, 763, and 789 of SEQ ID NO: 2 in the sequence listing. The use of sequences specific to this phyletic line



containing these characteristic nucleotides enables the design of probes having high specificity and gene amplification primers having high specificity and excellent amplification efficiency. For example, it becomes possible to design such a primer containing 15 or more continuous nucleotides (containing nucleotides that differ from those of closely related species so as to always contain positions that differ in terms of nucleotides from those of closely related species) of nucleotide sequences of the *gyrB* and *rpoD* genes, preferably containing 20 or more nucleotides, and further preferably containing 20 or more nucleotides and 40 or fewer continuous nucleotides of nucleotide sequences of the *gyrB* and *rpoD* genes. Similarly, it also becomes possible to design a probe containing 15 or more continuous nucleotides (containing nucleotides that differ from those of closely related species so as to always contain positions that differ in terms of nucleotides from those of closely related species) of nucleotide sequences of the *gyrB* and *rpoD* genes, preferably containing 20 or more nucleotides, and further preferably containing 20 or more nucleotides and 100 or fewer continuous nucleotides of nucleotide sequences of the *gyrB* and *rpoD* genes. Furthermore, to produce the primers and the probes, a region containing the above different nucleotides at high frequencies (containing 2 or more such nucleotides) can be preferably used, such as: in the case of the *gyrB* gene, a region containing positions 96 and 107, a region containing 2 or more positions of any of positions 258, 270, 279, or 285, a region containing 2 or more positions of any of positions 543, 552, or 557, a region containing 2 or more positions of any of positions 690, 702, or 714, and a region containing 2 or more positions of any of positions 729, 733, or 734, a region containing 759 and 771, and a region containing 2 or more positions of any of positions 782, 786, 792, or 795; and in the case of the *rpoD* gene, a region containing 2 or more positions of any of positions 66, 67, 75, or 90, a region containing 2 or more positions of any of positions 177, 178, 180, or 186, a region containing 2 or more positions of any of positions 223, 227, 228, or 231, a region containing 2 or more positions of any of positions 250, 251, 255, 257,

259, or 264, a region containing 2 or more positions of any of positions 300, 301, 302, 303, 305, 313, or 314, a region containing 2 or more positions of any of positions 362, 369, 373, 374, or 380, a region containing 2 or more positions of any of positions 400, 402, 409, 410, 415, or 416, a region containing 2 or more positions of any of positions 423, 427, 433, 444, or 447, a region containing 2 or more positions of any of positions 504, 510, or 513, a region containing 2 or more positions of any of positions 543, 556, or 558, and a region containing 2 or more positions of any of positions 747, 757, 762, or 763. Furthermore, in the case of primers, the 3'-terminus is preferably a nucleotide specific to *Vibrio cholerae* and *mimicus* bacterial groups. The present invention encompasses a kit for detecting, quantifying, or identifying *Vibrio cholera* and *mimicus* using these primers and probes in combination with other reagents.

In the meantime, *Vibrio cholerae* and *mimicus* bacterial groups, that is, the *Vibrio cholerae* bacterial group and the *Vibrio mimicus* bacterial group, further form an independent mono phyletic group (Fig. 1). Hence, to establish a genetic screening method that can separately detect the *Vibrio cholerae* and *mimicus* bacterial groups, regions that are conserved within each of the *Vibrio cholerae* and *mimicus* groups, and nucleotide positions that differ from each other within the same were identified in a manner similar to the above. Specifically, consensus sequences of phylogenetic groups to which *Vibrio cholerae* and *mimicus* belong were compared with each other, so that a phylogenetically-specific information map was produced (Figs. 4, 5, 6, and 7). Regarding the mono phyletic group to which *Vibrio cholerae* belongs, the following positions are specific: as shown in Fig. 4, in the case of the *gyrB* gene, positions 15, 36, 39, 42, 45, 48, 51, 90, 111, 133, 226, 285, 291, 306, 330, 384, 390, 399, 507, 708, 756, 837, 867, 873, 879, 882, and 885 of SEQ ID NO: 3 in the sequence listing; and as shown in Fig. 5, in the case of the *rpoD* gene, positions 12, 93, 96, 105, 114, 115, 116, 117, 126, 132, 141, 156, 198, 201, 216, 222, 231, 240, 252, 254, 255, 260, 261, 264, 276, 285, 291, 327, 333, 342, 345, 424, 426,

432, 441, 445, 446, 448, 450, 453, 468, 489, 495, 501, 519, 522, 525, 540, 549, 570, 585, 591, 600, 603, 606, 639, 645, 654, 657, 666, 675, 679, 680, 681, 687, 702, 705, 708, 714, 720, 723, 729, 732, 741, 750, 765, 768, 795, and 804 of SEQ ID NO: 4 in the sequence listing. Regarding the phyletic group to which *Vibrio mimicus* belongs, the following positions are specific: as shown in Fig. 6, in the case of the *gyrB* gene, positions 15, 36, 39, 42, 45, 48, 51, 90, 111, 133, 226, 285, 291, 306, 330, 384, 390, 399, 507, 708, 756, 837, 867, 873, 879, 882, and 885 of SEQ ID NO: 5 in the sequence listing; and as shown in Fig. 7, in the case of the *rpoD* gene, positions 12, 93, 96, 105, 114, 115, 116, 117, 126, 132, 141, 156, 198, 201, 216, 222, 231, 240, 252, 254, 255, 260, 261, 264, 276, 285, 291, 327, 333, 342, 345, 424, 426, 432, 441, 445, 446, 448, 450, 453, 468, 489, 495, 501, 519, 522, 525, 540, 549, 570, 585, 591, 600, 603, 606, 639, 645, 654, 657, 666, 675, 679, 680, 681, 687, 702, 705, 708, 714, 720, 723, 729, 732, 741, 750, 765, 768, 795, and 804 of SEQ ID NO: 6 in the sequence listing. The use of sequences that are specific to the phyletic group to which *Vibrio cholerae* or *Vibrio mimicus* belongs and containing these characteristic nucleotides enables the design of probes having high specificity and gene amplification primers having high specificity and excellent amplification efficiency. For example, it becomes possible to design a primer containing 15 or more continuous nucleotides (containing nucleotides that differ between the two bacterial species so as to always contain nucleotide positions that are different between *Vibrio cholerae* and *Vibrio mimicus*) of nucleotide sequences of the *gyrB* and *rpoD* genes, preferably containing 20 or more nucleotides, and further preferably containing 20 or more nucleotides and 40 or fewer continuous nucleotides of nucleotide sequences of the *gyrB* and *rpoD* genes. Similarly, it also becomes possible to design a probe containing 15 or more continuous nucleotides (containing nucleotides that differ between the 2 bacterial species so as to always contain nucleotide positions that differ between *Vibrio mimicus* and *Vibrio cholerae*) of nucleotide sequences of the *gyrB* and *rpoD* genes, preferably containing 20 or more nucleotides, and further

preferably containing 20 or more nucleotides and 100 or fewer continuous nucleotides of nucleotide sequences of the *gyrB* and *rpoD* genes. Furthermore, to produce the primers and the probes, a region containing the above different nucleotides at high frequencies (containing 2 or more such nucleotides) can be preferably used, such as: in the case of the *gyrB* gene, a region containing 2 or more positions of any of positions 36, 39, 42, 45, 48, or 51, a region containing 2 or more positions of any of positions 285, 291, or 306, a region containing 2 or more positions of any of positions 384, 390, or 399, and a region containing 2 or more positions of any of positions 867, 873, 879, 882, or 885; and in the case of the *rpoD* gene, a region containing 2 or more positions of any of positions 93, 96, 105, 114, 115, 116, or 117, a region containing 2 or more positions of any of positions 126, 132, or 141, a region containing 2 or more positions of any of positions 216, 222, 231, or 240, a region containing 2 or more positions of any of positions 252, 254, 255, 260, 261, or 264, a region containing 2 or more positions of any of positions 276, 285, or 291, a region containing 2 or more positions of any of positions 327, 333, 342, or 345, a region containing 2 or more positions of any of positions 424, 426, 432, 441, 445, or 446, a region containing 2 or more positions of any of positions 448, 450, 453, or 468, a region containing 2 or more positions of any of positions 489, 495, or 501, a region containing 2 or more positions of any of positions 519, 522, 525, or 540, a region containing 2 or more positions of any of positions 585, 591, 600, 603, or 606, a region containing 2 or more positions of any of positions 639, 645, 654, or 657, a region containing 2 or more positions of any of positions 666, 675, 679, 680, 681, or 687, a region containing 2 or more positions of any of positions 702, 705, 708, 714, 720, or 723, and a region containing 2 or more positions of any of positions 729, 732, 741, or 750. Furthermore, in the case of primers, the 3'-terminus is preferably a nucleotide specific to *Vibrio cholerae* or *mimicus*.

Specifically, primers for detecting, quantifying, or identifying the *Vibrio cholerae* and *mimicus* bacterial groups are as follows.

(1) In the case of the *gyrB* gene, examples of such gene amplification primers include: a gene amplification primer containing a strand or a complementary strand thereof of 15 or more continuous nucleotides of SEQ ID NO: 1 containing nucleotides at positions 96 and 107 of SEQ ID NO: 1; a gene amplification primer comprising a strand or a complementary strand thereof of 15 or more continuous nucleotides of SEQ ID NO: 1 containing 2 or more nucleotides at any of positions 258, 270, 279, or 285 of SEQ ID NO: 1; a gene amplification primer comprising a strand or a complementary strand thereof of 15 or more continuous nucleotides of SEQ ID NO: 1 containing 2 or more nucleotides at any of positions 543, 552, or 557 of SEQ ID NO: 1; a gene amplification primer comprising a strand or a complementary strand thereof of 15 or more continuous nucleotides of SEQ ID NO: 1 containing 2 or more nucleotides at any of positions 690, 702, or 714 of SEQ ID NO: 1; a gene amplification primer containing a strand or a complementary strand thereof of 15 or more continuous nucleotides of SEQ ID NO: 1 containing 2 or more nucleotides at any of positions 729, 733, or 734 of SEQ ID NO: 1; a gene amplification primer containing a strand or a complementary strand thereof of 15 or more continuous nucleotides of SEQ ID NO: 1 containing nucleotides at positions 759 and 771 of SEQ ID NO: 1; and a gene amplification primer containing a strand or a complementary strand thereof of 15 or more continuous nucleotides of SEQ ID NO: 1 containing 2 or more nucleotides at any of positions 782, 786, 792, or 795 of SEQ ID NO: 1. More specific examples include a gene amplification primer that is any one of 5'-tycaywscsaaacttacca-3' or a complementary strand corresponding thereto, 5'-gaaytctggcgtgtcgatcaag-3' or a complementary strand corresponding thereto, 5'-catrtagttgttcaaagtacgg-3' or a complementary strand corresponding thereto, 5'-ggatttyacytccgaagaaacyagc-3' or a complementary strand corresponding thereto, 5'-ygccagcttctcattcatr-3' or a complementary strand corresponding thereto, 5'-cgcttcgcttgggtttcc-3' or a complementary strand corresponding thereto, or 5'-caataatcttcgaacaaacgt-3' or a complementary strand corresponding thereto and

gene amplification primers that contain the strands or the complementary strands thereof comprising the above sequences.

(2) In the case of the *rpoD* gene, examples of such gene amplification primers include: a gene amplification primer containing a strand or a complementary strand thereof of 15 or more continuous nucleotides containing 2 or more nucleotides of SEQ ID NO: 2 at any of positions 66, 67, 75 or 90 of SEQ ID NO: 2; a gene amplification primer containing a strand or a complementary strand thereof of 15 or more continuous nucleotides of SEQ ID NO: 2 containing 2 or more nucleotides at any of positions 177, 178, 180, or 186 of SEQ ID NO: 2; a gene amplification primer containing a strand or a complementary strand thereof of 15 or more continuous nucleotides of SEQ ID NO: 2 containing 2 or more nucleotides at any of positions 223, 227, 228, or 231 of SEQ ID NO: 2; a gene amplification primer containing a strand or a complementary strand thereof of 15 or more continuous nucleotides of SEQ ID NO: 2 containing 2 or more nucleotides at any of positions 250, 251, 255, 257, 259, or 264 of SEQ ID NO: 2; a gene amplification primer containing a strand or a complementary strand thereof of 15 or more continuous nucleotides of SEQ ID NO: 2 containing 2 or more nucleotides at any of positions 300, 301, 302, 303, 305, 313, or 314 of SEQ ID NO: 2; a gene amplification primer containing a strand or a complementary strand thereof of 15 or more continuous nucleotides of SEQ ID NO: 2 containing 2 or more nucleotides at any of positions 362, 369, 373, 374, or 380 of SEQ ID NO: 2; a gene amplification primer containing a strand or a complementary strand thereof of 15 or more continuous nucleotides of SEQ ID NO: 2 containing 2 or more nucleotides at any of positions 400, 402, 409, 410, 415, or 416 of SEQ ID NO: 2; a gene amplification primer containing a strand or a complementary strand thereof of 15 or more continuous nucleotides of SEQ ID NO: 2 containing 2 or more nucleotides at any of positions 423, 427, 433, 444, or 447 of SEQ ID NO: 2; a gene amplification primer containing a strand or a complementary strand thereof of 15 or more continuous nucleotides of SEQ ID NO: 2 containing 2 or more

nucleotides at any of positions 504, 510, or 513 of SEQ ID NO: 2; a gene amplification primer containing a strand or a complementary strand thereof of 15 or more continuous nucleotides of SEQ ID NO: 2 containing 2 or more nucleotides at any of positions 543, 556, or 558 of SEQ ID NO: 2; and a gene amplification primer containing a strand or a complementary strand thereof of 15 or more continuous nucleotides of SEQ ID NO: 2 containing 2 or more nucleotides at any of positions 747, 757, 762, or 763 of SEQ ID NO: 2. More specific examples include a gene amplification primer that is any one of 5'-gattgctgagtatcctggaaccatc-3' or a complementary strand corresponding thereto, 5'-gaycctaacgacatggaaacc-3' or a complementary strand corresponding thereto, 5'-ttcwgarcytytctgaagcs-3' or a complementary strand corresponding thereto, 5'-agatgaygmkgctcgysgar-3' or a complementary strand corresponding thereto, 5'-cgacggtgaaagyagcgacag-3' or a complementary strand corresponding thereto, 5'-caatgaactgcgcggyaagtt-3' or a complementary strand corresponding thereto, 5'-gtcagaccaaattcattaac-3' or a complementary strand corresponding thereto, 5'-gyytgamgcttcagawgcttgrtka-3' or a complementary strand corresponding thereto, 5'-ygargtrcgcagagtttcaacc-3' or a complementary strand corresponding thereto, 5'-catyaccaarcgytcttgg-3' or a complementary strand corresponding thereto, or 5'-cgytcaacagacagtgawgtc-3' or a complementary strand corresponding thereto and gene amplification primers that contain the strands or the complementary strands thereof comprising the above sequences.

Primers for *Vibrio cholerae* are as follows.

(1) In the case of *gyrB*, examples of such gene amplification primers include: a gene amplification primer containing a strand or a complementary strand thereof of 15 or more continuous nucleotides of SEQ ID NO: 3 containing 2 or more nucleotides at any of positions 36, 39, 42, 45, 48, or 51 of SEQ ID NO: 3; a gene amplification primer containing a strand or a complementary strand thereof of 15 or more continuous nucleotides of SEQ ID NO: 3 containing 2 or more nucleotides at any of positions 285, 291, or 306 of SEQ ID NO: 3; a gene

amplification primer containing a strand or a complementary strand thereof of 15 or more continuous nucleotides of SEQ ID NO: 3 containing 2 or more nucleotides at any of positions 384, 390, or 399 of SEQ ID NO: 3; or a gene amplification primer containing a strand or a complementary strand thereof of 15 or more continuous nucleotides of SEQ ID NO: 3 containing 2 or more nucleotides at any of positions 867, 873, 879, 882, or 885 of SEQ ID NO: 3. More specific examples include a gene amplification primer comprising 5'-ggtggttaacgcgctytct-3' or a complementary strand corresponding thereto, a gene amplification primer comprising 5'-ycgatgaacgtgaagaagataaa-3' or a complementary strand corresponding thereto, a gene amplification primer comprising 5'-tgagaaagtcttccacttt-3' or a complementary strand corresponding thereto, a gene amplification primer comprising 5'-gttaaagtggaagactttc-3' or a complementary strand corresponding thereto, and a gene amplification primer comprising 5'-gggtaagccwgcaagatcc-3' or a complementary strand corresponding thereto and gene amplification primers that contain the strands or the complementary strands thereof comprising the above sequences.

(2) In the case of *rpoD*, examples of such gene amplification primers include: a gene amplification primer containing a strand or a complementary strand thereof of 15 or more continuous nucleotides of SEQ ID NO: 4 containing 2 or more nucleotides at any of positions 93, 96, 105, 114, 115, 116, or 117 of SEQ ID NO: 4; a gene amplification primer containing a strand or a complementary strand thereof of 15 or more continuous nucleotides of SEQ ID NO: 4 containing 2 or more nucleotides at any of positions 126, 132, or 141 of SEQ ID NO: 4; a gene amplification primer containing a strand or a complementary strand thereof of 15 or more continuous nucleotides of SEQ ID NO: 4 containing 2 or more nucleotides at any of positions 216, 222, 231, or 240 of SEQ ID NO: 4; a gene amplification primer containing a strand or a complementary strand thereof of 15 or more continuous nucleotides of SEQ ID NO: 4 containing 2 or more nucleotides at any of positions 252, 254, 255, 260,



261, or 264 of SEQ ID NO: 4; a gene amplification primer containing a strand or a complementary strand thereof of 15 or more continuous nucleotides of SEQ ID NO: 4 containing 2 or more nucleotides at any of positions 276, 285, or 291 of SEQ ID NO: 4; a gene amplification primer containing a strand or a complementary strand thereof of 15 or more continuous nucleotides of SEQ ID NO: 4 containing 2 or more nucleotides at any of positions 327, 333, 342, or 345 of SEQ ID NO: 4; a gene amplification primer containing a strand or a complementary strand thereof of 15 or more continuous nucleotides containing 2 or more nucleotides at any of positions 424, 426, 432, 441, 445, or 446 of SEQ ID NO: 4; a gene amplification primer containing a strand or a complementary strand thereof of 15 or more continuous nucleotides of SEQ ID NO: 4 containing 2 or more nucleotides at any of positions 448, 450, 453, or 468 of SEQ ID NO: 4; a gene amplification primer containing a strand or a complementary strand thereof of 15 or more continuous nucleotides of SEQ ID NO: 4 containing 2 or more nucleotides at any of positions 489, 495, or 501 of SEQ ID NO: 4; a gene amplification primer containing a strand or a complementary strand thereof of 15 or more continuous nucleotides of SEQ ID NO: 4 containing 2 or more nucleotides at any of positions 519, 522, 525, or 540 of SEQ ID NO: 4; a gene amplification primer containing a strand or a complementary strand thereof of 15 or more continuous nucleotides of SEQ ID NO: 4 containing 2 or more nucleotides at any of positions 585, 591, 600, 603, or 606 of SEQ ID NO: 4; a gene amplification primer containing a strand or a complementary strand thereof of 15 or more continuous nucleotides of SEQ ID NO: 4 containing 2 or more nucleotides at any of positions 639, 645, 654, or 657 of SEQ ID NO: 4; a gene amplification primer containing a strand or a complementary strand thereof of 15 or more continuous nucleotides of SEQ ID NO: 4 containing 2 or more nucleotides at any of positions 679, 680, 681, 687, or 702 of SEQ ID NO: 4; a gene amplification primer containing a strand or a complementary strand thereof of 15 or more continuous nucleotides of SEQ ID NO: 4 containing 2 or more

nucleotides at any of positions 705, 708, 714, 720, or 723 of SEQ ID NO: 4; and a gene amplification primer containing a strand or a complementary strand thereof of 15 or more continuous nucleotides of SEQ ID NO: 4 containing 2 or more nucleotides at any of positions 729, 732, 741, or 750 of SEQ ID NO: 4. More specific examples include a gene amplification primer comprising 5'-attcttgagcagtttgatcgt-3' or a complementary strand corresponding thereto, a gene amplification primer comprising 5'-caggccgaagagctacgtctc-3' or a complementary strand corresponding thereto, a gene amplification primer comprising 5'-tgagctttctgaagcggatctcgcg-3' or a complementary strand corresponding thereto, a gene amplification primer comprising 5'-gaagatgatgctgtcgtcgaa-3' or a complementary strand corresponding thereto, a gene amplification primer comprising 5'-gaagatgaagacgaagat-3' or a complementary strand corresponding thereto, a gene amplification primer comprising 5'-cggtatcgaccctgaactg-3' or a complementary strand corresponding thereto, a gene amplification primer comprising 5'-catcaagcttctgaagcgtcaga-3' or a complementary strand corresponding thereto, a gene amplification primer comprising 5'-tcaaccaagtggtcgaattgc-3' or a complementary strand corresponding thereto, a gene amplification primer comprising 5'-acggaagatatccarcactaa-3' or a complementary strand corresponding thereto, a gene amplification primer comprising 5'-gcgaacacgatccattgaagtg-3' or a complementary strand corresponding thereto, a gene amplification primer comprising 5'-gatgaacgatttcttcggcatc-3' or a complementary strand corresponding thereto, a gene amplification primer comprising 5'-aaggactttatccagccac-3' or a complementary strand corresponding thereto, a gene amplification primer comprising 5'-ttcttcttgctcacggactttcgc-3' or a complementary strand corresponding thereto, a gene amplification primer comprising 5'-ttctgaattgaacggcggatc-3' or a complementary strand corresponding thereto, and a gene amplification primer comprising 5'-tgtctcttgctcgatcatttgc-3' or a complementary strand corresponding thereto, and gene amplification primers that

contain the strands or the complementary strands thereof comprising the above sequences.

Primers for *Vibrio mimicus* are as follows.

(1) In the case of *gyrB*, examples of such gene amplification primers include: a gene amplification primer containing a strand or a complementary strand thereof of 15 or more continuous nucleotides of SEQ ID NO: 5 containing 2 or more nucleotides at any of positions 36, 39, 42, 45, 48, or 51 of SEQ ID NO: 5; a gene amplification primer containing a strand or a complementary strand thereof of 15 or more continuous nucleotides of SEQ ID NO: 5 containing 2 or more nucleotides at any of positions 285, 291, or 306 of SEQ ID NO: 5; a gene amplification primer containing a strand or a complementary strand thereof of 15 or more continuous nucleotides of SEQ ID NO: 5 containing 2 or more nucleotides at any of positions 384, 390, or 399 of SEQ ID NO: 5; and a gene amplification primer containing a strand or a complementary strand thereof of 15 or more continuous nucleotides of SEQ ID NO: 5 containing 2 or more nucleotides at any of positions 867, 873, 879, 882, or 885 of SEQ ID NO: 5. More specific examples include a gene amplification primer comprising 5'-ggtagtgaatgcctgtca-3' or a complementary strand corresponding thereto, a gene amplification primer comprising 5'-cggatgagcgtgaagaagataag-3' or a complementary strand corresponding thereto, a gene amplification primer comprising 5'-tgaaaaagtattccacttc-3' or a complementary strand corresponding thereto, a gene amplification primer comprising 5'-gttgaagtgaataactttt-3' or a complementary strand corresponding thereto, and a gene amplification primer comprising 5'-wggcaaaccagckarrtct-3' or a complementary strand corresponding thereto, and gene amplification primers that contain the strands or the complementary strands thereof comprising the above sequences.

(2) In the case of *rpoD*, examples of such gene amplification primers include: a gene amplification primer containing a strand or a complementary strand thereof of 15 or more continuous nucleotides of SEQ ID NO: 6 containing

2 or more nucleotides at any of positions 93, 96, 105, 114, 115, 116, or 117 of SEQ ID NO: 6; a gene amplification primer containing a strand or a complementary strand thereof of 15 or more continuous nucleotides of SEQ ID NO: 6 containing 2 or more nucleotides at any of positions 126, 132, or 141 of SEQ ID NO: 6; a gene amplification primer containing a strand or a complementary strand thereof of 15 or more continuous nucleotides of SEQ ID NO: 6 containing 2 or more nucleotides at any of positions 216, 222, 231, or 240 of SEQ ID NO: 6; a gene amplification primer containing a strand or a complementary strand thereof of 15 or more continuous nucleotides of SEQ ID NO: 6 containing 2 or more nucleotides at any of positions 252, 254, 255, 260, 261, or 264 of SEQ ID NO: 6; a gene amplification primer containing a strand or a complementary strand thereof of 15 or more continuous nucleotides of SEQ ID NO: 6 containing 2 or more nucleotides at any of positions 276, 285, or 291 of SEQ ID NO: 6; a gene amplification primer containing a strand or a complementary strand thereof of 15 or more continuous nucleotides of SEQ ID NO: 6 containing 2 or more nucleotides at any of positions 327, 333, 342, or 345 of SEQ ID NO: 6; a gene amplification primer containing a strand or a complementary strand thereof of 15 or more continuous nucleotides of SEQ ID NO: 6 containing 2 or more nucleotides at any of positions 424, 426, 432, 441, 445, or 446 of SEQ ID NO: 6; a gene amplification primer containing a strand or a complementary strand thereof of 15 or more continuous nucleotides of SEQ ID NO: 6 containing 2 or more nucleotides at any of positions 448, 450, 453, or 468 of SEQ ID NO: 6; a gene amplification primer containing a strand or a complementary strand thereof of 15 or more continuous nucleotides of SEQ ID NO: 6 containing 2 or more nucleotides at any of positions 489, 495, or 501 of SEQ ID NO: 6; a gene amplification primer containing a strand or a complementary strand thereof of 15 or more continuous nucleotides of SEQ ID NO: 6 containing 2 or more nucleotides at any of positions 519, 522, 525, or 540; a gene amplification primer containing a strand or a complementary strand thereof

of 15 or more continuous nucleotides of SEQ ID NO: 6 containing 2 or more nucleotides at any of positions 585, 591, 600, 603, or 606 of SEQ ID NO: 6; a gene amplification primer containing a strand or a complementary strand thereof of 15 or more continuous nucleotides of SEQ ID NO: 6 containing 2 or more nucleotides at any of positions 639, 645, 654, or 657 of SEQ ID NO: 6; a gene amplification primer containing a strand or a complementary strand thereof of 15 or more continuous nucleotides of SEQ ID NO: 6 containing 2 or more nucleotides at any of positions 679, 680, 681, 687, or 702 of SEQ ID NO: 6; a gene amplification primer containing a strand or a complementary strand thereof of 15 or more continuous nucleotides of SEQ ID NO: 6 containing 2 or more nucleotides at any of positions 705, 708, 714, 720, or 723 of SEQ ID NO: 6; and a gene amplification primer containing a strand or a complementary strand thereof of 15 or more continuous nucleotides of SEQ ID NO: 6 containing 2 or more nucleotides at any of positions 729, 732, 741, or 750 of SEQ ID NO: 6. More specific examples include a gene amplification primer comprising 5'-cattcttgaacagtttgacaag-3' or a complementary strand corresponding thereto, a gene amplification primer comprising 5'-caggcagaagaactacgtctg-3' or a complementary strand corresponding thereto, a gene amplification primer comprising 5'-agarctctctgaagccgatctcgct-3' or a complementary strand corresponding thereto, a gene amplification primer comprising 5'-gaagatgacgaggtcgcgag-3' or a complementary strand corresponding thereto, a gene amplification primer comprising 5'-gaggatgaagatgaagac-3' or a complementary strand corresponding thereto, a gene amplification primer comprising 5'-gggtattgaccctgagctc-3' or a complementary strand corresponding thereto, a gene amplification primer comprising 5'-taaccaagcatctgaagcttcaag-3' or a complementary strand corresponding thereto, a gene amplification primer comprising 5'-tcaaccaaattggtcaaattgt-3' or a complementary strand corresponding thereto, a gene amplification primer comprising 5'-gcggaaratatccagtaccag-3' or a complementary strand corresponding thereto, a gene amplification primer

comprising 5'-acgaacacgatccatcgaggta-3' or a complementary strand corresponding thereto, a gene amplification primer comprising 5'-aataaatgatttctttggcatt-3' or a complementary strand corresponding thereto, a gene amplification primer comprising 5'-gagyactttatcragccat-3' or a complementary strand corresponding thereto, a gene amplification primer comprising 5'-gtcttcttgctcagctacttttg-3' or a complementary strand corresponding thereto, a gene amplification primer comprising 5'-ttggattgaaggcgcaata-3' or a complementary strand corresponding thereto, and a gene amplification primer comprising 5'-agtctcytggtcgatcatctgm-3' or a complementary strand corresponding thereto, and gene amplification primers that contain the strands or the complementary strands thereof comprising the above sequences.

The above regions and strands or complementary strands thereof comprising the above sequences can be directly used as primers. Furthermore, if necessary, other sequences such as adaptor sequences can be contained in primers.

The present invention encompasses a kit for detecting, quantifying, or identifying *Vibrio cholerae* or *mimicus* using these primers and probes in combination with other reagents.

A gene amplification method used in the present invention is not limited to the PCR method. The primers and the probes can be used in a specific amplification method that is based on the specificity of primers or a method for specifically inhibiting amplification. Furthermore, the primers and the probes can also be similarly used in a quantification and amplification reaction using specific amplification primers and a labeled specific probe in combination. Furthermore, a probe sequence can also be used alone. A method for using the probes is not limited to one involving the solid phase or the liquid phase. The primers and the probes can also be utilized for carrying out real-time PCR using a reagent such as cyber green for detecting amplified double-stranded DNA or real-time PCR to which FRET or the like is applied.

The use of information on specificity between phyletic group (Figs. 2, 3,

and 4) of the nucleotide sequences obtained in the present invention enables the design of probes having high specificity and gene amplification primers having high specificity and excellent amplification efficiency. Table 2 shows the sequences of PCR primers produced based on specific nucleotide information regarding *Vibrio cholerae* or *Vibrio mimicus* (Figs. 2, 3, and 4) obtained in the present invention.

Table 2. Primers for specifically detecting *V. cholerae* and *V. mimicus*

Target gene	Primer	Sequence	Length	Position	Direction
<i>gyrB</i>	CMgF	gaaytctggcgtgctgatcaag	22	258-279	Sense
	CMgR	catrtagttgttcaaagtacgg	22	564-543	Antisense
<i>rpoD</i>	CMrF	gaycctaacgacatggaaacc	21	166-186	Sense
	CMrR	gtcacgaccaaattcattaac	21	420-400	Antisense

Information concerning phylogenetic groups has been obtained. Thus, it is possible to design specific primers or probes for almost all of the analyzed sequences.

The present invention will be hereafter described in detail by referring to examples. Each example is an embodiment of the present invention, and the present invention is not limited by these examples.

This specification includes part or all of the contents as disclosed in the specification or drawings of Japanese Patent Application No. 2002-362878, which is a priority document of the present application.

## BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows the result of molecular phylogenetic analysis after linking partial sequences of the *gyrB* and *rpoD* genes. This is a molecular phylogenetic tree produced by the neighbor-joining method, showing that *Vibrio cholerae* and *mimicus* belong to a mono phyletic group differing from those of other bacteria

belonging to the genus *Vibrio*. Furthermore, each of *Vibrio cholerae* and *Vibrio mimicus* forms an independent mono phyletic group.

Fig. 2 shows the results of determination of and comparison between the consensus sequence (upper case) of the *gyrB* gene of the cluster to which *Vibrio cholerae* and *mimicus* belong (shown in Fig. 1) and the consensus sequence (lower case) of the *gyrB* gene of closely related clusters (C1, 2, and 3 in Fig. 1). Sites denoted with “●” are nucleotides specific to *Vibrio cholerae* and *mimicus*.

Fig. 3 shows the results of determination of and comparison between the consensus sequence (upper case) of the *rpoD* gene of the cluster to which *Vibrio cholerae* and *mimicus* belong (shown in Fig. 1) and the consensus sequence (lower case) of the *rpoD* gene of the closely related clusters (C1, 2, and 3 in Fig. 1). Sites denoted with “●” are nucleotides specific to *Vibrio cholerae* and *mimicus*.

Fig. 4 shows the results of determination of and comparison between the consensus sequence (upper case) of the *gyrB* gene of the cluster to which *Vibrio cholerae* belongs (shown in Fig. 1) and the consensus sequence (lower case) of the *gyrB* gene of the cluster to which *Vibrio mimicus* belongs (shown in Fig. 1). Sites denoted with “●” are nucleotides specific to *Vibrio cholerae*.

Fig. 5 shows the results of determination of and comparison between the consensus sequence (upper case) of the *rpoD* gene of the cluster to which *Vibrio cholera* belongs (shown in Fig. 1) and the consensus sequence (lower case) of the *rpoD* gene of the cluster to which *Vibrio mimicus* belongs (shown in Fig. 1). Sites denoted with “●” are nucleotides specific to *Vibrio cholerae*.

Fig. 6 shows the results of determination of and comparison between the consensus sequence (upper case) of the *gyrB* gene of the cluster to which *Vibrio mimicus* belongs (shown in Fig. 1) and the consensus sequence (lower case) of the *gyrB* gene of the cluster to which *Vibrio cholerae* belongs (shown in Fig. 1). Sites denoted with “●” are nucleotides specific to *Vibrio mimicus*.

Fig. 7 shows the results of determination of and comparison between the



consensus sequence (upper case) of the *rpoD* gene of the cluster to which *Vibrio mimicus* belongs (shown in Fig. 1) and the consensus sequence (lower case) of the *rpoD* gene of the cluster to which *Vibrio cholerae* belongs (shown in Fig. 1). Sites denoted with “●” are nucleotides specific to *Vibrio mimicus*.

## BEST MODE OF CARRYING OUT THE INVENTION

### [Example 1]

An example of using gene amplification primers (shown in Table 2) designed and obtained according to the present invention using regions specific to *Vibrio cholerae* and *mimicus* is shown. In addition, primers described in claim 5 (2) and (3) and claim 11 (2) and (7) correspond to CMgF, CMgR, CMrF, and CMrR in Table 2, respectively. PCR was carried out using chromosomal DNAs as templates extracted from test strains, AmpliTaq Gold (PE Applied Biosystems), and a total of 20 µl of reaction solution. Regarding thermal cyclers conditions, after 10 minutes of heating at 95°C, 35 cycles of 94°C for 1 minute, 1 minute of annealing (see Table 5 for annealing temperatures), and 72°C for 1 minute were conducted, finally followed by 10 minutes of elongation reaction at 72°C. Samples obtained after reaction were subjected to 1% agarose gel electrophoresis, and then stained with ethidium bromide. The presence or the absence of amplified genes was confirmed under UV irradiation. Amplified products were confirmed only for the DNAs derived from the bacterial strains determined to belong to *Vibrio cholerae* and *mimicus* with both combinations of CMgF and CMgR targeting the *gyrB* gene and CMrF and CMrR targeting the *rpoD* gene (Table 6).

### [Example 2]

An example of using gene amplification primers (shown in Table 3 and Table 4) designed and obtained according to the present invention using regions specific to *Vibrio cholerae* or *mimicus* is shown. In addition, primers described

in claim 19 (1), (3), (4), and (5) and claim 25 (1) and (14) are specific to *Vibrio cholera*. These primers are described in CF1, CF2, CR2, CR1, CrF1, and CrR1 in Table 3. Primers described in claim 33 (1), (3), (4), and (5) and claim 39 (7) and (13) are specific to *Vibrio mimicus*. These primers correspond to MF1, MF2, MR2, MR1, MrF1, and MrR1 in Table 4, respectively.

In a manner similar to Example 1, PCR was carried out using as templates chromosomal DNAs extracted from test strains (see Table 5 for annealing temperatures). In all cases, the use of *Vibrio cholera*-specific primers resulted in detection of amplification products only from *Vibrio cholera* and the use of *Vibrio mimicus*-specific primers resulted in detection of amplification products only from *Vibrio mimicus* (Table 6).

Table 3. Primers for specifically detecting *V. cholerae*

Target gene	Primer	Sequence	Length	Position	Direction
<i>gyrB</i>	CF1	ggtgggtaacgcgctytct	19	33-51	Sense
	CR2	gttaaagtgaagactttc	19	402-384	Antisense
	CF2	tgagaaagtcttcacttt	19	381-399	Sense
	CR1	gggtaagccwgcaagatcc	19	885-867	Antisense
<i>rpoD</i>	CrF1	attcttgagcagttgatcgt	21	97-117	Sense
	CrR1	ttctgaattgaacggcggatc	21	725-705	Antisense

Table 4. Primers for specifically detecting *V. mimicus*

Target gene	Primer	Sequence	Length	Position	Direction
<i>gyrB</i>	MF1	ggtagtgaatgccctgtca	19	33-51	Sense
	MR2	gttgaagtgaataactttt	19	402-384	Antisense
	MF2	tgaaaaagtattccacttc	19	381-399	Sense
	MR1	wggcaaaccagckarrtet	19	885-867	Antisense
<i>rpoD</i>	MrF1	taaccaagcatctgaagcttcaag	24	423-446	Sense
	MrR1	gtcttcttgctcacgtactttttg	24	702-679	Antisense

Table 5. PCR conditions for primers for specifically detecting *V. cholerae* and *V. mimicus*

Target gene	Sense primer	Antisense primer	Amplification product (bp)	PCR conditions		
				Annealing temperature (°C)	Number of cycles	Primer temperature (μM)
<i>gyrB</i> ( <i>V. cholerae</i> , <i>V. mimicus</i> )	CMgF	CMgR	307	60	35	0.1
<i>rpoD</i> ( <i>V. cholerae</i> , <i>V. mimicus</i> )	CMrF	CMrR	255	60	35	0.1

<i>mimicus</i> )						
<i>gyrB</i> ( <i>V. cholerae</i> )	CF1	CR2	370	65	35	0.1
	CF2	CR1	505	60	35	0.1
<i>rpoD</i> ( <i>V. cholerae</i> )	CrF1	CrR1	629	65	35	0.1
<i>gyrB</i> ( <i>V. mimicus</i> )	MF1	MR2	370	65	35	0.1
	MF2	MR1	505	60	35	0.1
<i>rpoD</i> ( <i>V. mimicus</i> )	MrF1	MrR1	280	65	35	0.1

Table 6

Serial No.	Name	Strain name	Serotype	Cholerae toxin gene	PCR							
					CMgF & CMgR	CMrF & CMrR	CF1 & CR2	CF2 & CR1	CrF1 & CrR1	MF1 & MR2	MF2 & MR1	MrF1 & MrR1
1	<i>V. vulnificus</i>	ATCC 27562 T			-	-	-	-	-	-	-	-
2	<i>V. vulnificus</i>	ATCC 29306			-	-	-	-	-	-	-	-
3	<i>V. vulnificus</i>	ATCC 29307			-	-	-	-	-	-	-	-
4	<i>V. vulnificus</i>	ATCC 33147			-	-	-	-	-	-	-	-
5	<i>V. vulnificus</i>	ATCC 33148			-	-	-	-	-	-	-	-
6	<i>V. vulnificus</i>	ATCC 33149			-	-	-	-	-	-	-	-
7	<i>V. vulnificus</i>	ATCC 33814			-	-	-	-	-	-	-	-
8	<i>V. vulnificus</i>	ATCC 33815			-	-	-	-	-	-	-	-
9	<i>V. vulnificus</i>	ATCC 33816			-	-	-	-	-	-	-	-
10	<i>V. vulnificus</i>	ATCC 33817			-	-	-	-	-	-	-	-
11	<i>V. vulnificus</i>	ATCC 43382			-	-	-	-	-	-	-	-
12	<i>V. vulnificus</i>	ATCC BAA-86			-	-	-	-	-	-	-	-
13	<i>V. vulnificus</i>	ATCC BAA-87			-	-	-	-	-	-	-	-
14	<i>V. vulnificus</i>	ATCC BAA-88			-	-	-	-	-	-	-	-
15	<i>V. vulnificus</i>	ATCC BAA-89			-	-	-	-	-	-	-	-
16	<i>V. vulnificus</i>	ATCC BAA-90			-	-	-	-	-	-	-	-
17	<i>V. vulnificus</i>	JCM 3726			-	-	-	-	-	-	-	-
18	<i>V. vulnificus</i>	JCM 3727			-	-	-	-	-	-	-	-
19	<i>V. vulnificus</i>	JCM 3728			-	-	-	-	-	-	-	-
20	<i>V. vulnificus</i>	JCM 3729			-	-	-	-	-	-	-	-
21	<i>V. vulnificus</i>	JCM 3730			-	-	-	-	-	-	-	-

22	<i>V. vulnificus</i>	JCM 3731			-	-	-	-	-	-	-	-
23	<i>V. vulnificus</i>	No. 4			-	-	-	-	-	-	-	-
24	<i>V. vulnificus</i>	No. 9			-	-	-	-	-	-	-	-
25	<i>V. vulnificus</i>	No. 66			-	-	-	-	-	-	-	-
26	<i>V. vulnificus</i>	No. 74			-	-	-	-	-	-	-	-
27	<i>V. vulnificus</i>	No. 81			-	-	-	-	-	-	-	-
28	<i>V. vulnificus</i>	No. 130			-	-	-	-	-	-	-	-
29	<i>V. vulnificus</i>	No. 196			-	-	-	-	-	-	-	-
30	<i>V. vulnificus</i>	No. 202			-	-	-	-	-	-	-	-
31	<i>V. vulnificus</i>	No. 496			-	-	-	-	-	-	-	-
32	<i>V. vulnificus</i>	No. 965			-	-	-	-	-	-	-	-
33	<i>V. cholerae</i>	DU1	monO1	+	+	+	+	+	+	+	-	-
34	<i>V. cholerae</i>	DU2	O1	+	+	+	+	+	+	+	-	-
35	<i>V. cholerae</i>	DU3	O1	+	+	+	+	+	+	+	-	-
36	<i>V. cholerae</i>	DU6	O1	+	+	+	+	+	+	+	-	-
37	<i>V. cholerae</i>	DU19	O1	+	+	+	+	+	+	+	-	-
38	<i>V. cholerae</i>	DU63	monO1	+	+	+	+	+	+	+	-	-
39	<i>V. cholerae</i>	DU81	O1	+	+	+	+	+	+	+	-	-
40	<i>V. cholerae</i>	DU94	O1	+	+	+	+	+	+	+	-	-
41	<i>V. cholerae</i>	T2	monO1	+	+	+	+	+	+	+	-	-
42	<i>V. cholerae</i>	T6	monO1	+	+	+	+	+	+	+	-	-
43	<i>V. cholerae</i>	T97	monO1	+	+	+	+	+	+	+	-	-
44	<i>V. cholerae</i>	T98	O1	+	+	+	+	+	+	+	-	-
45	<i>V. cholerae</i>	T116	monO1	+	+	+	+	+	+	+	-	-
46	<i>V. cholerae</i>	NM20	monO1	+	+	+	+	+	+	+	-	-
47	<i>V. cholerae</i>	NM26	O1	+	+	+	+	+	+	+	-	-
48	<i>V. cholerae</i>	NM48	O1	+	+	+	+	+	+	+	-	-
49	<i>V. cholerae</i>	NM67	monO1	+	+	+	+	+	+	+	-	-
50	<i>V. cholerae</i>	NM84	monO1	+	+	+	+	+	+	+	-	-
51	<i>V. cholerae</i>	NM85	monO1	+	+	+	+	+	+	+	-	-
52	<i>V. cholerae</i>	Q37	O1	+	+	+	+	+	+	+	-	-
53	<i>V. cholerae</i>	Q57	O1	+	+	+	+	+	+	+	-	-
54	<i>V. cholerae</i>	Q59	monO1	+	+	+	+	+	+	+	-	-
55	<i>V. cholerae</i>	Q66	monO1	+	+	+	+	+	+	+	-	-
56	<i>V. cholerae</i>	Q70	monO1	+	+	+	+	+	+	+	-	-
57	<i>V. cholerae</i>	Q90	monO1	+	+	+	+	+	+	+	-	-
58	<i>V. cholerae</i>	K47	monO1	+	+	+	+	+	+	+	-	-
59	<i>V. cholerae</i>	OPC24	monO1	+	+	+	+	+	+	+	-	-
60	<i>V. cholerae</i>	OPC39	monO1	+	+	+	+	+	+	+	-	-
61	<i>V. cholerae</i>	OPC60	O1	+	+	+	+	+	+	+	-	-
62	<i>V. mimicus</i>	ATCC 33563 T			-	+	+	-	-	-	+	+
63	<i>V. mimicus</i>	ATCC 33654			-	+	+	-	-	-	+	+
64	<i>V. mimicus</i>	ATCC 33655			+	+	+	-	-	-	+	+
65	<i>V. mimicus</i>	ATCC 700326			+	+	+	-	-	-	+	+

66	<i>V. diazo-Trophicus</i>	ATCC 33466 T			-	-	-	-	-	-	-	-
67	<i>V. navarrensis</i>	ATCC 51183 T			-	-	-	-	-	-	-	-
68	<i>V. metschnikovii</i>	ATCC 700040 T			-	-	-	-	-	-	-	-
69	<i>V. cincinnatiensis</i>	ATCC 35912 T			-	-	-	-	-	-	-	-
70	<i>V. ordalii</i>	NCIMB 2167 T			-	-	-	-	-	-	-	-
71	<i>Listonella anguillarum</i>	NCIMB 6 T			-	-	-	-	-	-	-	-
72	<i>V. alginolyticus</i>	IFO 15630 T			-	-	-	-	-	-	-	-
73	<i>V. campbellii</i>	IFO 15631 T			-	-	-	-	-	-	-	-
74	<i>V. carchariae</i>	IFO 15632 T			-	-	-	-	-	-	-	-
75	<i>V. harveyi</i>	IFO 15634 T			-	-	-	-	-	-	-	-
76	<i>V. nereis</i>	IFO 15637 T			-	-	-	-	-	-	-	-
77	<i>V. parahaemolyticus</i>	IFO 12711 T			-	-	-	-	-	-	-	-
78	<i>V. proteolyticus</i>	IFO 13287 T			-	-	-	-	-	-	-	-
79	<i>V. tubiashii</i>	IFO 15644 T			-	-	-	-	-	-	-	-
80	<i>Vibrio</i> sp.	V11			-	-	-	-	-	-	-	-
81	<i>Vibrio</i> sp.	V41			-	-	-	-	-	-	-	-
82	<i>Shewanella</i> sp.	V52			-	-	-	-	-	-	-	-
83	<i>Vibrio</i> sp.	V65			-	-	-	-	-	-	-	-
84	<i>Vibrio</i> sp.	V70			-	-	-	-	-	-	-	-
85	<i>V. hollisae</i>	ATCC 33564 T			-	-	-	-	-	-	-	-

All publications, patents and patent applications cited herein are incorporated herein by reference in their entirety.

#### Industrial Applicability

The *gyrB* and *rpoD* gene primers and probes of the present invention have been designed based on the understanding of the phylogenetic relationship of *Vibrio cholerae* and *Vibrio mimicus*. Thus, the primers and the probes have been studied for improving specificity and are excellent in terms of detection accuracy. Hence, the primers and the probes are advantageous when direct detection is carried out under circumstances where a bacterium is not isolated from foods, clinical specimens, or the like that are contaminated with closely related bacterial species.